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International application number: PCT/AU04/001762

International filing date: 16 December 2004 (16.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: AU
Number: 2003906979
Filing date: 16 December 2003 (16.12.2003)

Date of receipt at the International Bureau: 17 January 2005 (17.01.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

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# NOVEL BINDING DOMAIN

## Field of the invention

The present invention relates to binding reagents derived from cytokine binding domains (CBDs) and their use as affinity reagents, diagnostic reagents, therapeutic reagents and as protein scaffolds.

## Background to the invention

Antibodies are the paradigm of specific high-affinity binding reagents and provide an antigen binding site by interaction of variable heavy ($V_H$) and variable light ($V_L$) immunoglobulin domains. The binding interface is formed by six surface polypeptide loops, termed complementarity determining regions (CDRs), three from each variable domain, which are highly variable and combined provide a sufficiently large surface area for interaction with antigen. Specific binding reagents can be formed by association of only the $V_H$ and $V_L$ domains into an Fv module. Bacterial expression is enhanced by joining the V-domains with a linker polypeptide into a single-chain scFv molecule.

## Summary of the invention

We have developed novel binding reagents based on cytokine-binding domains (CBDs). CBDs consist of two linked Fibronectin type III (FnIII) domains (each an Ig-like fold (Leahy DJ *et al.*, 1992, Science 258: 987-991). We have compared the CBDs of a large number of known tertiary structures to reveal a number of common structural features that indicate that these domains will form an ideal framework for the design of molecules that would be useful as diagnostic reagents or therapeutics directed to particular molecular targets, and particularly those targets associated with clinical disease.

These CBDs are known to bind their target molecules primarily at the juncture of the two FnIII-like domains (the cytokine hinging region), engaging their target molecules by loops on the outer elbow of the two domains of the CBD. These loops are similar to the CDR loops found on the antigen-binding surface of antibody variable domains, however, the association between loops from the two domains in a CBD exhibit important differences to antibody CDR loop association. In antibody variable domains these loops from the heavy chain associate in parallel with those of the light chain, however, the cytokine binding loops (CBL) of the CBD binding regions form a linear association (see Figure 1). We describe herein the novel use of the CBD framework to design and generate binding reagents that will interact with a target molecule through the loops forming the CBD cytokine binding region. Such binding reagents will have a variety of uses, as affinity reagents including diagnostic and therapeutic uses and applications.

Accordingly, in a first aspect, the present invention provides a binding reagent comprising an extracellular cytokine binding domain (CBD), wherein at least one solvent exposed region comprises a modification which alters the binding characteristics of the domain.

Preferably, the CBD is derived from the extracellular domain of a growth factor/cytokine receptor family member. More preferably, the CBD is derived from the extracellular domain of a growth factor/cytokine receptor family member listed in Figure 5. More preferably, the CBD is derived from the extracellular domain of a receptor selected from IL-2 receptor, IL-3 receptor, IL-4 receptor, IL-5 receptor, IL-6 receptor, IL-7 receptor, IL-9 receptor, IL-11 receptor, IL-12 receptor, IL-13 receptor, IL-15 receptor and IL-21 receptor, G-CSF receptor, GM-CSF receptor,. LIF receptor, oncostatin M receptor, cardiotrophin CT-1 receptor, ciliary neutrotrophic factor (CNTF) receptor, prolactin receptor, leptin receptor, erythropoietin receptor, growth hormone receptor, cytokine receptor-like factor 1, .class 1 cytokine receptor, thymic stromal lymphopoietin protein receptor or gp130.

Typically the solvent-exposed region is a cytokine binding region, preferably a surface exposed loop.

Preferably, the size and/or area of the solvent exposed region is altered as compared with the corresponding solvent exposed region in the unmodified CBD.

In a preferred embodiment, the affinity of the modified CBD for at least one natural ligand of the unmodified CBD is reduced or abolished.

In another embodiment, the binding specificity of the modified CBD is different to that of the unmodified CBD. Preferably, the unmodified CBD is derived from the extracellular domain of a first receptor having specificity for a first ligand, the cytokine binding loops of the unmodified CBD have been replaced with the cytokine binding loops of a second receptor having specificity for a second ligand, and the modified CBD has specificity for the second ligand. In a preferred embodiment the first receptor is IL-6 receptor and the second receptor is either prolactin receptor, LIF receptor or oncostatin M receptor, thus altering the ligand specificity of the CBD from IL-6 to either prolactin and/or growth hormone or LIF or oncostatin M, respectively.

In an alternative further preferred embodiment, the first CBD is prolactin receptor, or IL-11 receptor, or CNTF receptor which has been modified so that the solvent-exposed regions have been replaced with the second cytokine receptor solvent exposed regions which alters the specificity of the first CBD.

Other modifications may be made to the CBDs. For example, the binding reagent may further comprise one or more modifications to the hinge region of the CBD and/or the binding reagent may further comprises one or more modifications to the binding interface of the FnIII-like domains of the CBD.

In one embodiment, the binding reagent lacks disulphide-bond forming cysteine residues. This is advantageous where the binding reagent is designed to function in an intracellular environment.

In a preferred embodiment, cysteine residues have been removed or replaced within the binding reagent. In a further preferred embodiment, cysteine residues have been removed or replaced in the binding reagent only in one FnIII-like domain whilst maintaining the cysteine residues in the other FnIII-like domain unaltered.

The binding reagents of the invention may be linked to other molecules, for example by covalent or non-covalent means. In preferred embodiments, the binding reagents (CBD) of the invention may be linked (without restriction) to molecules such as enzymes, drugs, lipids, sugars, nucleic acids and viruses.

In one embodiment, the binding reagent may contain solvent exposed cysteine residues for the site-specific attachment of other entities.

In a related aspect, the present invention also provides a multivalent reagent comprising two or more binding reagents of the invention.

In one embodiment, two or more CBDs may be joined together into multimers through either covalent linkage or non-covalent linkage or a combination of linkages, including the use of chemical or genetically-encoded linkers. The CBDs of the multimers may possess the same, or different target specificities thus providing multivalent or multispecific reagents. In a preferred embodiment two CBDs may be joined to form a dimer through either covalent linkage or non-covalent linkage or a combination of linkages thereby providing two target binding affinities. It will be appreciated that in cases where the two CBDs in the dimer have the same target specificity, the dimer will be bivalent and will have increased avidity or functional affinity for the target molecule.

In one embodiment, the binding reagent or multivalent reagent is immobilised on a solid support or coupled to a biosensor surface.

The present invention also provides a polynucleotide encoding a binding reagent or multivalent reagent of the invention, as well as nucleic acid vectors comprising such polynucleotides and host cells comprising said polynucleotides and/or vectors.

Binding reagents of the invention may be used therapeutically, for example to target receptors and/or to compete with naturally occurring ligands. Accordingly, the present invention also provides a pharmaceutical composition comprising a binding reagent of the invention and a pharmaceutically acceptable carrier or diluent.

The present invention also provides a method of treating a pathological condition in a subject, which method comprises administering to the subject a binding reagent of the invention.

In a second aspect, the present invention provides a method of selecting a binding reagent with an affinity for a target molecule which comprises

(i) providing a plurality of polynucleotides encoding binding reagents comprising a CBD, which polynucleotides comprise one or more modifications in regions encoding at least one solvent exposed region of the CBDs;

(ii) expressing the binding reagents encoded by the polynucleotides; and

(iii) selecting one or more binding reagents having an affinity for the target molecule.

In one embodiment, the plurality of nucleotides are randomised and/or have been subjected to mutagenesis, such as site-directed mutagenesis.

In a preferred embodiment, the binding reagents are expressed as a fusion protein linked to a phage coat protein and the screening process uses phage display. In an alternative embodiment, the screening process uses ribosomal display.

The target molecule may be a cytokine receptor ligand.

The binding reagents of the invention may also be used as a scaffold to display peptides. Libraries of such scaffold molecules may be used to screen for peptides having an activity of interest.

Accordingly, in a third aspect, the present invention provides a nucleic acid sequence encoding a peptide display scaffold comprising:

a) a first scaffold sequence encoding a CBD; and

b) a second sequence encoding a peptide and inserted at a site located in a region of said first scaffold sequence encoding a solvent-exposed region of the tertiary structure of said CBD.

Preferably, the second sequence substantially replaces the region of the first scaffold sequence encoding the solvent-exposed region.

Preferably, the CBD is derived from the extracellular domain of a growth factor/cytokine receptor.

Typically the solvent-exposed region is a cytokine binding region, preferably a surface exposed loop.

In one embodiment the nucleic acid sequence comprises a plurality of second sequences inserted into a plurality of sites. Thus the nucleic acid sequence encodes a peptide display scaffold comprising a) a first scaffold sequence encoding a CBD and b) a number of separately inserted sequences in which each sequence encodes a peptide and is inserted at a site located in a region of said first scaffold sequence encoding a solvent-exposed region of the tertiary structure of said CBD.

The present invention also provides an expression vector comprising a nucleic acid sequence of the third aspect of the invention.

In a related aspect, the present invention provides a CBD display library, comprising a plurality of expression vectors comprising nucleic acid sequences of the third aspect of the invention.

In a fourth aspect, the present invention provides an expression vector comprising:

a) a first nucleic acid sequence encoding a CBD;

b) an insertion site in a region between the ends of the first nucleic acid sequence, which region encodes a solvent-exposed region in the tertiary structure of said CBD, the insertion site comprising a nucleotide sequence unique to said expression vector which is cleaved by a type II restriction endonuclease and which allows a second nucleic acid sequence encoding an amino acid sequence to be inserted into the first nucleic acid to encode a modified CBD; and

c) a regulatory control sequence operably linked to said first nucleic acid sequence which directs expression of the first nucleic acid sequence.

The present invention also provides an expression vector comprising:

a) a first nucleic acid sequence encoding a CBD, said sequence comprising a deletion in a region which encodes a solvent-exposed region in the tertiary structure of said CBD;

b) an insertion site in place of the deleted sequence which site allows a second nucleic acid sequence encoding an amino acid sequence to be inserted into the first nucleic acid to encode a modified CBD.

c) a regulatory control sequence operably linked to said first nucleic acid sequence which directs expression of the first nucleic acid sequence.

In a related aspect, the present invention provides an expression vector comprising:

a) a first nucleic acid sequence encoding a CBD;

b) a number of insertion sites between the ends of the first nucleic acid sequence, which encodes a number of solvent-exposed regions in the tertiary structure of said CBD, each insertion site comprising a nucleotide sequence unique to said expression vector which is cleaved by a type II restriction endonuclease and which allows a nucleic acid sequence encoding an amino acid sequence to be inserted into the first nucleic acid to encode a modified CBD.

The present invention further provides a polypeptide encoded by the nucleic acid sequence of the third aspect of the invention, as well as a protein multimer comprising at least two of said polypeptides.

In a fifth aspect, the present invention provides a method of identifying a modified CBD which binds to a target molecule of interest, which method comprises:

(i) providing a CBD display library of the invention;

(ii) expressing the polypeptides encoded by the polynucleotides; and

(iii) selecting one or more polypeptides that bind to the target molecule.

Description of the Figures

**Figure 1**

(a) A coil representation of the backbone of a CBD, illustrated by the CBD of IL-6 receptor (D2 representing the N-terminal domain (cyan), and D3 representing the C-terminal domain (white)). The red and orange loops, L1 to L4 and L5 to L7 respectively, represent the loops from the N-terminal and C-terminal domains of the CBD that can engage a target macromolecule. (b) the view with the molecule rotated 90 degrees vertically looking on-fast to the seven loops.

(c) A coil representation of the backbone of the variable domains of the heavy (cyan) and light (white) chain of the Fv domain of an immunoglobulin, illustrated by the NC10 anti-neuraminidase Fv domain, showing the Fv domain's respective antigen binding CDRs in red and orange loops. (d) the view with the molecule rotated 90deg vertically looking on-fast to the seven loops.

Both molecules are draw to scale, and it can been seen that while the Fv antigen binding site is approximately isotropic in distributions, the CBD loops are long and narrow, offering a different type of surface topology when compare to the potential binding site of antibody molecules.

**Figure 2.** (a) A ribbon diagram of the CBD of IL-6R, showing the β-sheet arrangement of the two FnIII domains, and the Cytokine binding loops L1 to L7 (in red). The two β-sheets of D2 are coloured green and yellow, and that of D3 are coloured blue and cyan. (b) the same as in (a) but rotated 90° with the loops facing up.

**Figure 3.** The sequence of Interleukin-6 receptor extra-cellular domains, showing the CBD comprising domain D2 in dark grey, and domain D3 in lighter grey. Black horizontal arrows, red, orange, blue and cyan represent residues in the β-sheets of these domains, loops in the cytokine binding region, conserved residues in all known CBDs, the mainly conserved hydrophobic residues, and majority conserved residues in all known CBDs respectively.

**Figure 4.** The CBD of IL-6R with domain D2 coloured cyan, and domain D3 coloured white, with the loop residues from D2 and D3 coloured red and orange respectively. (a) and (c) have CPK and loop representations of the cytokine binding region loops L1 to L7: (b) and (d) are the same as in (a) and (c) but rotated 90° with the loops facing up.

**Figure 5.** Comparison of the sequences of CBDs from 77 known genes. Residues are coloured yellow, green, blue, brown, magenta and red to represent conserved cysteine, hydrophobic, hydroxylated (Y,T,S and including H), proline/glycine, acids and bases. In red double-headed arrows are the location of the 7 binding loops.

**Figure 6.** The backbone of the CBD of Interleukin-6 receptor, with the cytokine binding loops L1 to L7 coloured red. In (a) and (b) a CKP representation the residues (orange) that are conserved in all known CBDs. In (c) and (d) including a CPK representation of all residues which are almost always conserved and mainly hydrophobic (blue).

**Figure 7.** 3D alignment of CBD of IL6R (pink) to prolactin receptor (green), with loop regions from prolactin receptor (blue) chosen for grafting.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in molecular biology and biochemistry). Standard techniques are used for molecular and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd ed. (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4$^{th}$ Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

Cytokine binding domains

A CBD is defined herein as a polypeptide consisting of two domains, each domain having immunoglobulin folds in a fibronectin type III-like association of beta sheets. The two domains lie on a similar plane and are typically connected at about 90° to each other. Preferably, at least one domain comprises a tryptophan-arginine ladder region, which preferably comprises a WSXWS motif or variant thereof which forms a left-handed $3_{10}$ helix.

The two domains each comprise a number of solvent exposed regions, such as solvent exposed loops, that are arranged in a substantially linear manner over the two domains to form a binding region.

The structural definition referred to above can be illustrated with reference to the three dimensional structure of the IL-6 receptor, as described in Varghese JN *et al.*, 2002, PNAS 99(25):15959-15964 and PCT/AU02/01255 which also provide the atomic coordinates of the extracellular domain of the IL-6 receptor. The amino acid sequence of

IL-6R is given in Figure 3, which also highlights the location of various structures in the primary sequence. The CBD of IL-6 is defined by the D2 and D3 domains (amino acids 95 to 297). The two domains lie on a similar plane to form a long flat structure in which the D2 and D3 domains are connected at about 90° to each other. The D2 domain comprises 4 solvent exposed loops (L1: S106 to N110; L2: K133 to P138; L3: A160 to F168; and L4: Q190 to G193) and the D3 domain comprises 3 solvent exposed loops (L5: S227 to R233; L6: M250 to H256; and L7: Q276 to Q281), which together form a long and narrow binding area held in place by the rigid D2 and D3 framework of the CBD. The location of these loops in the three-dimensional structure of folded IL-6R is shown in Figure 4.

R239, F246, R237, W287, R274, W284 and Q276 together form the tryptophan-arginine ladder region, which comprises a WSXWS motif.

These various structural features are shown in Figures 2, 4 and 6, including the arrangement of beta sheets, the orientation of each of the two domains with respect to one another and the location of the solvent exposed loops.

The alignment of CBDs present in over seventy gene products is shown in Figure 5. It is clear from this alignment and structural information that is available for a number of these domains, that the CBD structure exemplified by IL-6R is conserved in other CBDs. Thus, CBDs can be defined with reference to the three-dimensional structure of domains D2 and D3 of IL-6R, in particular with reference to the structural coordinates of the backbone carbon atoms of IL-6R as provided in Varghese JN *et al.*, 2002 PNAS 99(25):15959-15964 and PCT/AU02/01255. However, it will be appreciated that the three-dimensional structure of other CBDs will not correspond precisely to that of the IL-6 receptor.

Figure 6 illustrates in the context of the IL-6 receptor, the regions of the CBD structure that are most highly conserved in known naturally occurring CBDs.

The binding reagents of the present invention are typically derived from naturally occurring CBDs, which are generally found in the extracellular domain of cytokine receptors and associated protein. Examples of naturally occurring CBDs are the CBD of the following proteins: interleukin receptors, such as IL-2 receptor, IL-3 receptor, IL-4 receptor, IL-5 receptor, IL-6 receptor, IL-7 receptor, IL-9 receptor, IL-11 receptor, IL-12 receptor, IL-13 receptor, IL15 receptor and IL21- receptor, G-CSF receptor, GM-CSF receptor, prolactin receptor, LIF receptor, oncostatin M receptor, cardiotrophin CT-1 receptor, ciliary neutrotrophic factor CNTF receptor, leptin receptor, erythropoietin receptor, gp130 and growth hormone receptor. The numbering of the amino acid residues that constitute the CBD for many of these proteins is provided in Figure 5.

However, in one embodiment of the binding reagents of the invention these CBDs have been modified in one or more solvent exposed regions to alter the binding

characteristics of the CBD. The solvent exposed region is typically a region of the domain that normally contacts the natural ligand for the CBD and is generally a surface exposed loop. For example, domains D2 and D3 of IL-6R together comprise 7 cytokine binding loops (L1 to L7), as described above. The location of these loops in other CBDs is shown in Figure 5. Thus it is preferred that modifications are made to one or more of these loop regions, or the equivalent regions in other CBDs.

Modifications can include insertions, deletions and/or substitutions. Typically, at least 2, preferably at least 3, 4 or 5, more preferably at least 10 amino acids of a solvent exposed region are altered. In one embodiment modifications are made to alter the size and/or area of the region, preferably to increase the size and/or area of the region, e.g. by at least 2, 3, 4 or 5 amino acids more preferably by at least 10 or 20 amino acids. Modifications can be made to any of the L1, L2, L3, L4, L5, L6 and L7 loops. For example, loop L1 in IL-6R is positioned in the center of the CBD (Figures 1, 2, 4 and 6). Since CBD of IL-6R L1 contains a natural disulphide bond, this might constrain the flexibility and so form an ideal semi-rigid scaffold for the display of larger, protruding 'finger-like' loops by insertion of additional amino acids within the L1 loop. These protruding 'finger-like' loops are then likely to provide a complementary binding surface to receptor cavities within the target antigen (protein) to which the CBD is capable of binding, analogous to the protruding loops observed in natural camelid VhH and shark NAR domains (Muyldermans S *et al.*, 2001 Trends Biochem Sci. 26(4):230-5) and (Nuttall SD *et al.*, 2000 Curr Pharm Biotechnol. 1(3):253-63).

Modifications can be made to a number of solvent exposed regions, preferably at least two or three different solvent exposed regions, e.g. to at least two or three of the L1, L2, L3, L4, L5, L6 and L7 loops. The solvent exposed regions can be modified by variation of the loop lengths or by variation in the amino acid sequence within the natural unmodified loops or by both strategies. Variation in the amino acid sequence of the natural unmodified loop can be achieved by designing the encoding gene to produce either specific point mutations or by random 'window' mutagenesis to randomise the entire loop sequences during the construction of a library repertoire. Variation in loop length may be achieved by designing the encoding gene to remove some of the amino acids in the CBD loops, thus making shorter loops or conversely by increasing the number of amino acids to extend the loops. These designs can be applied to two or more loops selected from L1, L2, L3, L4, L5, L6 and L7 loops. Alternatively the entire gene repertoire comprising the CBD framework and the randomised loops can be constructed using synthetic oligonucleotide primers.

As mentioned above, the object of such modifications is to alter the binding characteristics of the CBD. For example, the binding affinity of the CBD for at least one of its natural ligands can be reduced or abolished. Preferably at least a two-fold, more

preferably at least a five- or ten-fold reduction in binding affinity for at least one natural ligand is achieved.

One approach to modification of the CBDs is to alter the binding specificity for a different cytokine. In particular, this can be achieved by replacing the solvent exposed cytokine binding regions of a first CBD, which has specificity for a first cytokine, with the solvent exposed cytokine binding regions of a second CBD which has specificity for a second cytokine. For example, the loops L1 to L7 of the CBD of IL-6R could be replaced by loops L1 to L7 of the CBD of IL-11R to provide the modified binding reagent with specificity for IL-11 instead of IL-6. Similarly, the loops L1 to L7 of the CBD of IL-6R could be replaced by loops L1 to L7 of the CBD of prolactin receptor, LIF receptor or oncostatin M receptor to provide the modified binding reagent with specificity for prolactin and/or growth hormone, LIF or oncostatin M respectively instead of IL-6.

Modifications to binding reagents of the invention can be made using standard cloning techniques known to persons skilled in the art, such as site-directed mutagenesis.

In an embodiment of the invention, two or more CBDs may be joined together to form multimers. CBD multimers are one preferred design for therapeutic reagents since they have the potential to provide increased avidity and slower blood clearance rates which may provide favourable pharmacokinetic and biodistribution properties. The CBDs may be joined together through either covalent linkage or non-covalent linkage or a combination of linkages, including the use of chemical or genetically-encoded linkers. These types of linkers are well known to persons skilled in the art, for example in relation to antibodies and antibody fragments joined by chemicals. (Casey JL *et al.,* 2002 Br J Cancer. 86(9):1401-10), by the use of genetically-encoded linker polypeptides (BITE's scFv-scFv), or by the use of adhesive fusion-domains (Plückthun, A., and Pack, P 1997. Immunotechnology 3, 83-105). Indeed, two fibronectin type III-like domains from different CBDs may be cross-paired using linker polypeptides to form tightly-associated CBD multimers in the manner of a diabody (an antibody Fv dimer) or triabody (antibody Fv trimer) or tetrabody (antibody Fv tetramer) (Power BE et al., 2001, Cancer Immunol Immunother. 50(5):241-50). The resulting CBD multimers from any of these linker strategies described above may possess the same, or different target specificities thus providing multivalent or multispecific reagents. If two or more CBDs in the multimer have the same target specificity, the CBD multimer may have increased avidity (functional affinity) when binding to two or more target molecules. CBD multimers may be designed to have increased stability by modification to the interface contact regions, either through chemical or genetic alterations. For example, detailed examination of the CBD framework regions at the multimer interface may direct introduction of residue mutations or chemical modifications that stabilise the interface and thereby direct the preferential formation of CBD multimers. In one embodiment, the mutations are introduced to

interface residues other than F134(D2), F168 (D2) and H261 (D3). In another embodiment, the mutation is introduced at residue C174 (D2), C192 (D2) or C258 (D3).

Another approach to obtaining binding reagents having a binding affinity for a target molecule of interest is to produce libraries of polynucleotides which encode different binding reagents of the invention comprising modifications in one or more solvent-exposed regions and screen the libraries for binding to the target molecule using standard techniques such as phage display or ribosomal display. This screening approach will be described in more detail below.

Modifications can also be made to regions of the CBD in addition to the solvent exposed regions described above. For example, modifications can be made to the binding interface between the two FnIII-like domains (e.g. Pro107, Leu195 and Pro197 of D2 of IL-6R and Trp225, Leu232, Ala275, Pro200 and Pro222 of D3 of IL-6R are buried hydrophobic residues that are generally highly conserved and stabilise the association of D2 and D3). Such modifications include cysteine residue insertions to provide for disulphide stabilisation or other modifications that provide for improved stability at the domain interface including interface mutations that improve surface complementarity. Other modifications may be made to improve solubility and/or expression yield. These may include removal of cysteine residues not involved in disulphide bond formation within D2 and D3 (e.g. Cys174-Ser, Cys192-Ser, Cys258-Ser) to improve solubility of the IL-6R CBD framework.

Further modifications that can be made include modifications to the binding interface between the two Fn-like domains to alter the geometry of the spatial relationship between the two domains (e.g. the buried hydrophobic residues listed above for IL-6R). This in turn can be used to alter the orientation and/or association of the solvent exposed binding regions which will modify the characteristics/topology of the overall binding surface.

Where the binding reagents are designed to function in an intracellular context, it may be desirable to either remove disulphide bond-forming cysteines or replace disulphide bond-forming cysteines with amino acids such as alanine or serine.

Binding reagents of the invention can also be designed *de novo* based on the structural constraints for a CBD described above.

Binding reagents of the invention can be linked to other molecules, typically by covalent or non-covalent means. For example, binding reagents may be produced as fusion proteins, linked to other polypeptide sequences. Fusion partners can include enzymes, detectable labels and/or affinity tags for numerous diagnostic applications or to aid in purification. Fusion partners, without restriction, may be GFP (green fluorescent protein), GST (glutathione S-transferase), thioredoxin or hexahistidine. Other fusion partners include targeting sequences that direct binding reagents to particular sub-cellular

locations or direct binding reagents to extracellular locations e.g. secretion signals. In a preferred embodiment binding reagents of the invention do not comprise other regions of the receptor/protein from which they are derived i.e. any fusion partners are heterologous to the CBD.

Non-peptide molecules that can be linked to the binding reagents of the invention include pharmacologically active substances such as toxins or prodrugs, nucleic acids, such as inhibitory nucleic acids or nucleic acids encoding polypeptides, molecules that enhance the *in vivo* stability or lipophilic behaviour of the binding reagents such as PEG, and detectable labels such as dyes or chromophores or fluorophores or other imaging reagents.

Binding reagents of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. Binding reagents of the invention may also be in a substantially purified form, in which case they will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a binding reagent of the invention.

Binding reagents may also be immobilised to a solid phase, such as a substantially planar surface (e.g. a chip or a microtitre plate) or beads. Techniques for immobilising polypeptides to a solid phase are known in the art. In addition, where libraries of binding reagents are used (e.g. in screening methods), arrays of binding reagents immobilised to a solid phase can be produced (Lee YS and Mrksich, M, 2002 Trends Biotechnol. 20(12 Suppl):S14-8. and references contained therein).

Scaffold polypeptides

In another embodiment of the invention, the binding reagents of the invention function as a protein scaffold with other polypeptide sequences being inserted into solvent-exposed regions of the binding reagent for display on the surface of the scaffold. Such scaffolds may, for example, serve as a convenient means to present peptides in a conformationally constrained manner. The scaffolds may be used to produce CBDs with altered binding specificities and also to produce and/or screen for binding reagents having specificity for any target molecule of interest.

Heterologous polypeptide sequences may be inserted into one or more solvent exposed regions of the binding reagent, said solvent exposed regions being defined in the previous section. The CBD of the binding reagent functions as a protein scaffold for the inserted heterologous sequences, displaying the heterologous sequences on the surface of the binding reagent.

The heterologous sequences may replace all or part of the solvent exposed region of the CBD into which they are inserted, or may simply form additional sequence. Preferably, a plurality of heterologous sequences are inserted into a plurality of solvent exposed regions.

The heterologous sequences may be derived from the solvent exposed regions of another CBD. They may also be derived from other non-CBD molecules or be partially of fully randomised.

Other modifications can be made to the scaffold proteins of the invention as described in the previous section in relation to CBDs any they may also be linked to other molecules and/or produced as multimers as described above.

Production of binding reagents

Binding reagents of the invention may be made by chemical or recombinant means. Techniques for chemically synthesising peptides are reviewed by Borgia and Fields, 2000, TibTech 18: 243-251 and described in detail in the references contained therein. Typically binding reagents of the invention are made by recombinant means. Accordingly, the present invention provides polynucleotides encoding binding reagents of the present invention.

Polynucleotides of the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by a host cell or using an *in vitro* transcription/translation system, i.e. the vector is an expression vector. The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence

"operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

Vectors of the invention may be transformed or transfected into a suitable host cell to provide for expression of a binding reagent of the invention. This process may comprise culturing a host cell transformed with an expression vector under conditions to provide for expression by the vector of a coding sequence encoding the binding reagent, and optionally recovering the expressed binding reagent.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used, for example, to transfect or transform a host cell.

Control sequences operably linked to sequences encoding the protein of the invention include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell for which the expression vector is designed to be used in. The term "promoter" is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in prokaryotic or eukaryotic cells. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner or, alternatively, a tissue-specific manner. They may also be promoters that respond to specific stimuli. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the binding reagent can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

In a number of embodiments of the present invention, heterologous sequences are inserted into the binding reagents of the present invention, for example where the binding reagents are used as scaffold sequences. Such modifications are generally made by manipulating polynucleotides of the invention encoding binding reagents of the invention. This may conveniently be achieved by providing cloning vectors that comprise a sequence

encoding a CBD which sequence comprises one or more unique insertion sites in one or more regions encoding a solvent exposed region of said cytokine domain, to allow for easy insertion of nucleotide sequences encoding heterologous sequences into the appropriate regions of the CBD.

Each "unique" insertion site typically contains a nucleotide sequence that is recognised and cleaved by a type II restriction endonuclease, the nucleotide sequence not being present elsewhere in the cloning vector such that the cloning vector is cleaved by the restriction endonuclease only at the "unique" insertion site. This allows for easy insertion of nucleotide sequences having the appropriate ends by ligation with cut vector using standard techniques well know by persons skilled in the art. Preferably the insertion site is engineered – i.e. where the CBD is derived from a naturally occurring sequence, the insertion site does not naturally occur in the natural sequence.

Vectors and polynucleotides of the invention may be introduced into host cells for the purpose of replicating the vectors/polynucleotides and/or expressing the bind reagent proteins of the invention encoded by the polynucleotides of the invention. Host cells include prokaryotic cells such as bacterial cells and eukaryotic cells including yeast, fungi, insect cells and mammalian cells.

Vectors/polynucleotides of the invention may introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where vectors/polynucleotides of the invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

Host cells comprising polynucleotides of the invention may be used to express proteins of the invention. Host cells are cultured under suitable conditions which allow for expression of the binding reagents of the invention. Expression of the binding reagents may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG, or inducible expression may achieved through heat-induction, thereby denaturing the repressor and initiating protein synthesis.

Binding reagents of the invention can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption.

Libraries of binding reagents

Binding reagents of the present invention may be provided as libraries comprising a plurality of binding reagents which have different sequences in one or more solvent exposed regions. These libraries can typically be used in screening methods to identify a binding reagent with an activity of interest, such as affinity for a specific target molecule of interest.

Libraries of binding reagents are conveniently provided as libraries of polynucleotides encoding the binding reagents. The polynucleotides are generally mutagenised or randomised to produce a large number of different sequences which differ at one or more positions within at least one solvent exposed region.

Mutations can be introduced using a variety of techniques known in the art, such as site-directed mutagenesis. A number of methods for site-directed mutagenesis are known in the art, from methods employing single-stranded phage such as M13 to PCR-based techniques (see "PCR Protocols: A guide to methods and applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (eds.). Academic Press, New York, 1990). Another technique is to use the commercially available "Altered Sites II *in vitro* Mutagenesis System" (Promega - U.S. Patent N° 5,955,363). Techniques for site-directed mutagenesis are described above. Pluralities of randomly mutated sequences can be made by introducing mutations into a nucleotide sequence or pool of nucleotide sequences 'randomly' by a variety of techniques *in vivo,* including; using 'mutator strains', of bacteria such as *E. coli mutD5* (Low *et al.*, 1996, J Mol Biol 60: 9-68); and using the antibody hypermutation system of B-lymphocytes (Yelamos *et al.*, 1995, Nature 376: 225-9). Random mutations can also be introduced both *in vivo* and *in vitro* by chemical mutagens, and ionising or UV irradiation (Friedberg *et al.*, 1995, DNA repair and mutagenesis. SM Press, Washington D.C.), or incorporation of mutagenic base analogues (Zaccolo *et al.*, 1996 J Mol Biol 255: 589-603). 'Random' mutations can also be introduced into genes *in vitro* during polymerisation for example by using error-prone polymerases (Leung *et al.*, 1989, Technique 1: 11-15).

It is generally preferred to use mutagenesis techniques that vary the sequences present in the solvent-exposed binding regions of the CBD without modifying the remainder of the binding reagent. One method for accomplishing this is to provide a plurality of relatively short nucleotide sequences that are partially or fully mutagenised/randomised and clone these sequences into specific insertion sites in the binding reagent, as described above in relation to scaffold sequences.

Another approach is to synthesise a plurality of random synthetic oligonucleotides and then insert the oligonucleotides into a sequence encoding the binding reagent and/or replace a sequence encoding the binding reagent with the random synthetic

oligonucleotides. A suitable method is described in WO97/27213 where degenerate oligonucleotides are produced by adding more than one nucleotide precursor to the reaction at each step. The advantage of this method is that there is complete control over the extent to which each nucleotide position is held constant or randomised. Furthermore, if only C, G or T are allowed at the third base of each codon, the likelihood of producing premature stop codons is significantly reduced since two of the three stop codons have an A at this position (TAA and TGA).

Oligonucleotide synthesis is performed using techniques that are well known in the art (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, IRL Press at Oxford University Press 1991). Libraries can also be specified and purchased commercially. The synthetic process can be performed to allow the generation of all or most possible combinations over the length of the nucleic acid, thus generating a library of randomised nucleic acids. These randomised sequences are synthesised such that they allow in frame expression of the randomised peptide with any fusion partner.

In one embodiment, the library is fully randomised, with no sequence preferences or constants at any position. In another embodiment, the library is biased, i.e. partially randomised in which some positions within the sequence are either held constant, or are selected from a limited number of possible variations. Thus some nucleic acid or amino acid positions are kept constant with a view to maintaining certain structural or chemical characteristics.

The randomised oligonucleotides can then be inserted into a suitable site and/or replace a suitable sequence encoding a binding reagent.

Generally the library of sequences will be large enough such that a structurally diverse population of random sequences is presented. This ensures that a large subset of shapes and structures is represented and maximises the probability of a functional interaction.

It is preferred that the library comprises at least 1000 different nucleotide sequences, more preferably at least $10^4$, $10^5$ or $10^6$ different sequences. Preferably at least 5, 10, 15 or 20 amino acid residues of the peptides encoded by the nucleotide sequences are randomised.

Typically, the inserted peptides encoded by the randomised nucleotide sequences comprise at least 5, 8, 10 or 20 amino acids. In one embodiment, they also comprise fewer than 50, 30 or 25 amino acids.

The libraries of polynucleotides encoding binding reagents can be screening using any suitable technique to identify a binding reagent having an activity of interest. For example, to identify a binding reagent that binds to a target molecule of interest, the library of polynucleotides is incubated under conditions that allow for expression of the binding reagent polypeptides encoded by the polynucleotides and binding of the

polypeptides to the target molecule assessed. Binding is typically assessed *in vitro* or using whole cell assays.

Suitable techniques for screening the library for binding reagents having an activity of interest include phage display and ribosome display as well as the use of viral vectors, such as retroviral vectors.

The sequence of binding reagents identified in the screen can conveniently be determined using standard DNA sequencing techniques.

Diagnostic/Therapeutic Uses of Binding Reagents

Binding reagents of the invention, including those identified in the screening methods of the invention, may be used in methods of diagnosis/therapy by virtue of their specific binding to a target molecule of interest. Such uses will be analogous to the plethora of diagnostic/therapeutic applications already known in relation to antibodies and fragments thereof. For example, binding reagents of the invention may be used to detect the presence or absence of molecules of interest in a biological sample.

For diagnostic purposes, it may be convenient to immobilise the binding reagent to a solid phase, such as a dipstick, microtitre plate or chip.

As discussed above, binding reagents of the invention when used diagnostically will typically be linked to a diagnostic reagent such as a detectable label to allow easy detection of binding events *in vitro* or *in vivo*. Suitable labels include radioisotopes, dye markers or other imaging reagents for *in vivo* detection and/or localisation of target molecules.

Binding reagents may also be used therapeutically. For example, binding reagents may be used to target ligands that bind to extracellular receptors, such as cytokine receptors, and consequently antagonise the effect of such ligands. Cytokines and their receptors are involved in a wide range of disease processes and consequently modulation of their activity with specifically designed binding reagents based on CBDs has clear clinical implications.

In addition, binding reagents of the invention may be used, in a similar manner to antibodies, to target pharmacologically active substances to a cell of interest, such as a tumour cell, by virtue of binding to a cell surface molecule present specifically on the tumour cell to which the binding reagent binds specifically.

Administration

Binding reagents of the invention including binding reagents identified by the screening methods of the invention may preferably be combined with various components to produce compositions of the invention. Preferably the compositions are combined with

a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition of the invention may be administered by direct injection. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration. Typically, each protein may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

Polynucleotides/vectors encoding binding reagents may be administered directly as a naked nucleic acid construct. When the polynucleotides/vectors are administered as a naked nucleic acid, the amount of nucleic acid administered may typically be in the range of from 1 μg to 10 mg, preferably from 100 μg to 1 mg.

Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

Preferably the polynucleotide or vector of the invention is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, oral, intraocular or transdermal administration.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The various features and embodiments of the present invention, referred to in individual sections above apply, as appropriate, to other sections, *mutatis mutandis*. Consequently features specified in one section may be combined with features specified in other sections, as appropriate.

**EXAMPLE 1: Design of modified IL-6R CBD with altered binding specificity**

A PSI_BLAST search of the Brookhaven protein data bank revealed several structures that are closely related to the cytokine binding modules of the human IL-6 receptor. Of these the human prolactin receptor (PRLR) bound to human growth hormone was the most closely related structure that did not have overlapping specificity for interleukin-6. The binding of human growth hormone by the prolactin receptor is

mediated by the same loop framework as the cytokine binding modules of IL-6R use to bind IL-6.

*Sequence alignment*

The sequences of IL-6R and PRLR have been aligned according to their three dimensional structure using the MALIGN3D function of MODELLER6v2.

*Loop Definition*

Resides from the prolactin receptor in contact with human growth hormone were selected using VMD. VMD is a visualisation package developed at the University of Illinois which allows the viewing and manipulation of large molecules (Schwieters (2001) Journal of Magnetic Resonance 149:239-244). Loop regions were selected to contain these residues and residues which support the correct side-chain orientation of the contact residues.

*Homology modelling*

The sequence of a CBD binding reagent protein incorporating the framework residues of IL-6R and loop residues from the prolactin receptor was created. An initial series of homology models of the CBD binding reagent was generated using MODELLER6v2 with IL-6R framework residues and prolactin receptor loop residues as templates (see Figure 7). Model quality was assessed using PROCHECK. The loop regions were then refined ab initio using MODELLER6v2. Final model was then energy minimised and assessed for stability using CNS (Brünger AT *et al.*, 1998 Acta Crystallog D54:905-921).

**EXAMPLE 2: Production of an IL-6R CBD (binding reagent)**

Oligonucleotide primers were designed to amplify the CBD domains (the D2 and D3 domains) of human IL-6R by PCR, using IL-6R DNA as a template for this reaction. These PCR fragments of correct size and DNA sequence were cloned into pPOW5 bacterial expression vector. Protein expression was performed using eight different bacterial cell strains. One particular clone was selected for further stability and characterisation studies.

**EXAMPLE 3: Modification of an IL-6R CBD to introduce prolactin binding specificity**

In another gene construct the surface loops of prolactin receptor were grafted onto the IL-6R framework to produce a reagent with prolactin binding specificity. The grafting process involved replacement of seven solvent-exposed surface loops L1 to L7 of IL-6R by the equivalent loop residues from prolactin receptor, thereby effectively changing the binding specificity of the modified CBD from IL-6 to prolactin. There are several methods that can result in loop grafting and, in this example, the grafting process involved redesigning the gene encoding the modified IL-6R CBD such that the encoded surface loops L1 to L7 were that of prolactin receptor. The modified CBD gene was then constructed using a gene assembly process using synthetic oligonucleotides, typically 80 bases in length, which were assembled by hybridisation and ligation, into a section of double-stranded DNA encoding the entire modified CBD gene, in an overlapping "brick-laying" fashion. PCR and oligonucleotide primers were used as the final step to amplify the fully assembled gene. The DNA sequence of the PCR product was confirmed, and the modified CBD gene then sub-cloned and expressed in bacteria.

**EXAMPLE 4: Producing a novel binding reagent with modified intra-domain disulphide bonds**

We produced a binding reagent with a modified intra-domain disulphide bond. We used PCR to introduce a mutation at Cys174 to Ser on the CBD framework. This Cys174 in D2, usually forms a disulphide bond with another cysteine in D1, and is not involved with the D2 and D3 CBD associations. We have expressed this C174S mutant in bacteria.

**EXAMPLE 5: Producing a novel binding reagent with no cysteine residues in the D3 domain.**

We introduced another CBD framework mutation Cys258 to Serine in domain D3. This is a buried cysteine residue, mutated in an attempt to increase expression and stability of the CBD framework, and to ascertain whether the D3 domain could fold without the need for this Cysteine residue. We have expressed the CBD containing this D3 mutation in bacteria.

Clones isolated from the D3 library also contain this Cys258 to Ser framework mutation (see Examples 7 and 8).

**EXAMPLE 6: Producing a novel binding reagent with a removed (replaced) cysteine residues in the solvent exposed region.**

We noticed that when the PRLR loop graft onto the IL-6R framework was expressed in bacteria, there were less protein aggregates. There is a solvent exposed Cys192 in the IL-6R framework/loop junction, that is not involved in disulphide bond formation, which is not a cysteine residue in the equivalent position of the PRLR loop. Another mutation Cys192 to Ser, which lies at this framework/loop junction has been designed within D2 domain of IL-6R. This is a solvent exposed cysteine in the IL-6R framework and this mutation is expected to improve solubility of the IL-6R framework CBD.

**EXAMPLE 7: Producing a repertoire of novel binding reagents based on the CBD scaffold**

A gene library comprising the IL-6R CBD was constructed with mutations in the solvent-exposed surface loops. In this example, loops L5, L6, L7 were mutated in the D3 domain of the CBD by constructing a gene repertoire using overlapping synthetic oligonucleotides and the gene assembly techniques described in Example 3. In this example, the oligonucleotides contained flanking framework residues of IL-6R, then genetic diversity in the loops residues, followed by more framework residues. The genetic diversity encoding the amino acid residues in the loops, was biased in such a way as to reduce the chance of stop codons and also to encode for all 20 amino acids at each at each position of each loop. This diversity was achieved during the synthesis of the degenerate oligonucleotides, wherein instead of adding one nucleotide per position at a time, all four nucleotides (G, A, T and C) were added per position. Stop codons triplets usually end with an A e.g. TAA. The chance of this occurring in the degenerate oligonucleotide is reduced by only allowing G, T and C at the third position of the triplet. In order to make this library of genetic diversity, two different lengths of oligonucleotides were used. The oligonucleotides covering the loop regions were about 80 bases in length (top strand). The reverse oligonucleotide "cementing the bricks" were short, covering only the framework residues, about 55 bases in length. PCR was used to fill-in the gaps on the bottom strand. The cloned gene repertoire in the phagemid vector was transformed into bacterial competent cell. Several well-spaced isolated colonies were picked and grown in liquid culture, from which the DNA was extracted and sequenced. The DNA sequence from one of these isolated clones, has shown an altered loop 6 with residues in that loop replaced with new residues.

The CBD framework used for all the library constructions contains three mutations, Cys174 to Ser, Cys192 to Ser and Cys258 to Ser. In the D3 library, not only are there

changes in the loop residues but there is also a framework change Cys258 to Ser. The clone mentioned above has been expressed in bacteria.

1) partial DNA sequence of IL-6R D3 (loops 6 and 7 in bold, and Cys258)

R S K T F T T W M V K D **L Q H H C** V I H D A W S G L R H V V
**Q L R A Q E E F G Q G E W S E W**

2) partial DNA sequence of D3 library clone, showing changes in loop 6 and C258S

R S K T F T T W **A Q S R W Q** H H **S** V I H D A W S G L R H V V
**Q L R A Q E E F G Q G E W S E W**

**EXAMPLE 8: Producing a novel binding reagent with multi-loop mutations**

Another clone was isolated from the D3 library as described in Example 7 (previous example) and DNA sequencing showed changes in both loop 6 and loop 7 residues of the D3 domain. This clone, also containing a CBD framework mutation at Cys258 to Ser, has also been expressed in bacteria.

a) partial DNA sequence of IL-6R D3 (loops 6 and 7 in bold, and Cys258)

R S K T F T T W M V K D **L Q H H C** V I H D A W S G L R H V V
**Q L R A Q E E F G Q G E W S E W**

b) partial DNA sequence of D3 library clone, showing changes in loop 6, 7 and C258S

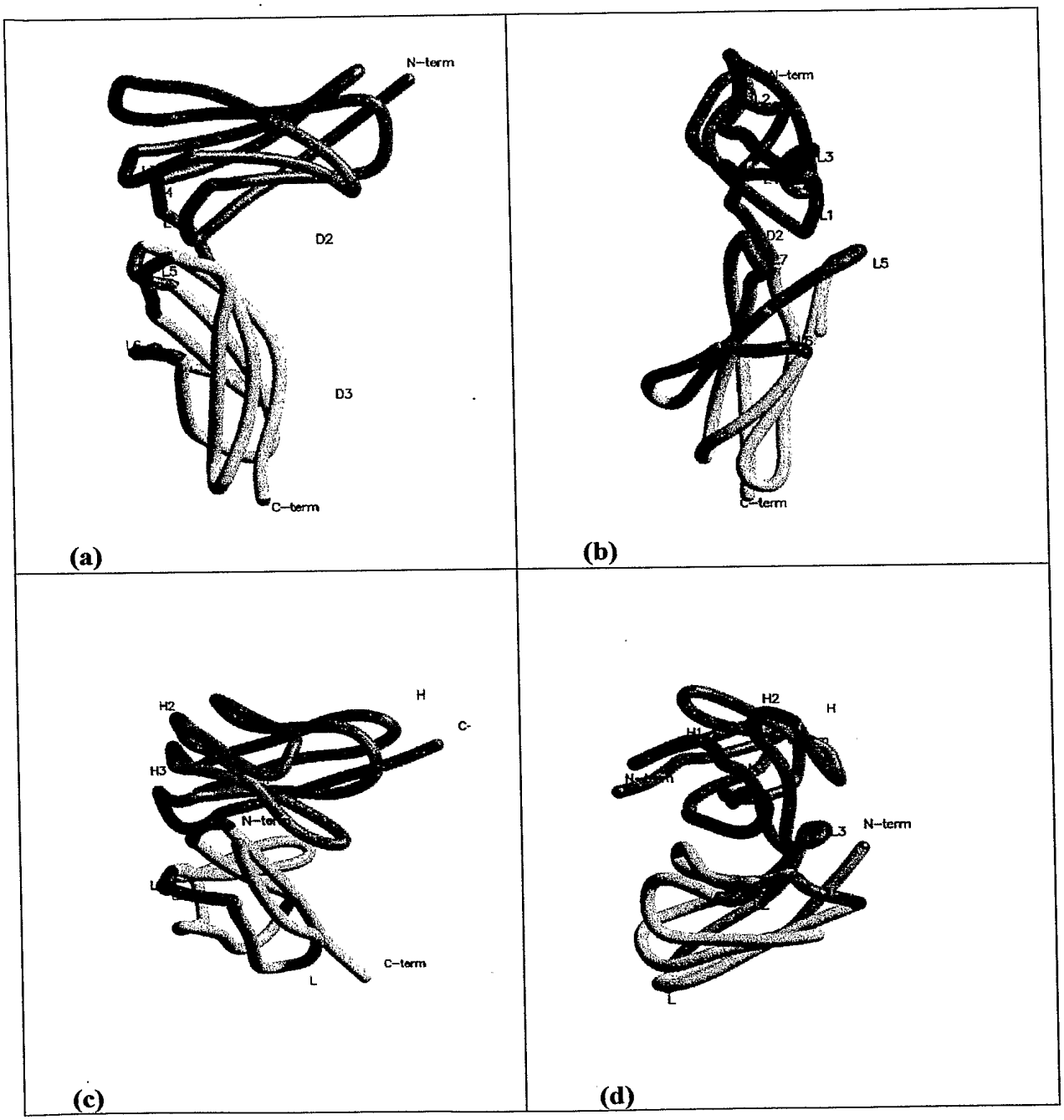
R S K T F T T W **S R Q N D Q** H H **S** V I H D A W S G L R H V V
**Q L R A R N E V R V G E** W S E W

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are readily apparent to those skilled in molecular biology or related fields are intended to be within the scope of the invention.

Dated this sixteenth day of December 2003

Commonwealth Scientific and Industrial
Research Organisation
Patent Attorneys for the Applicant:

F B RICE & CO


N-term
D2
D3
C-term
(a)
N-term
L3
L1
D2
L5
C-term
(b)
H2
H
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H3
N-ter
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(c)
H2
H
N-term
L3
L
(d)

**Figure 1**

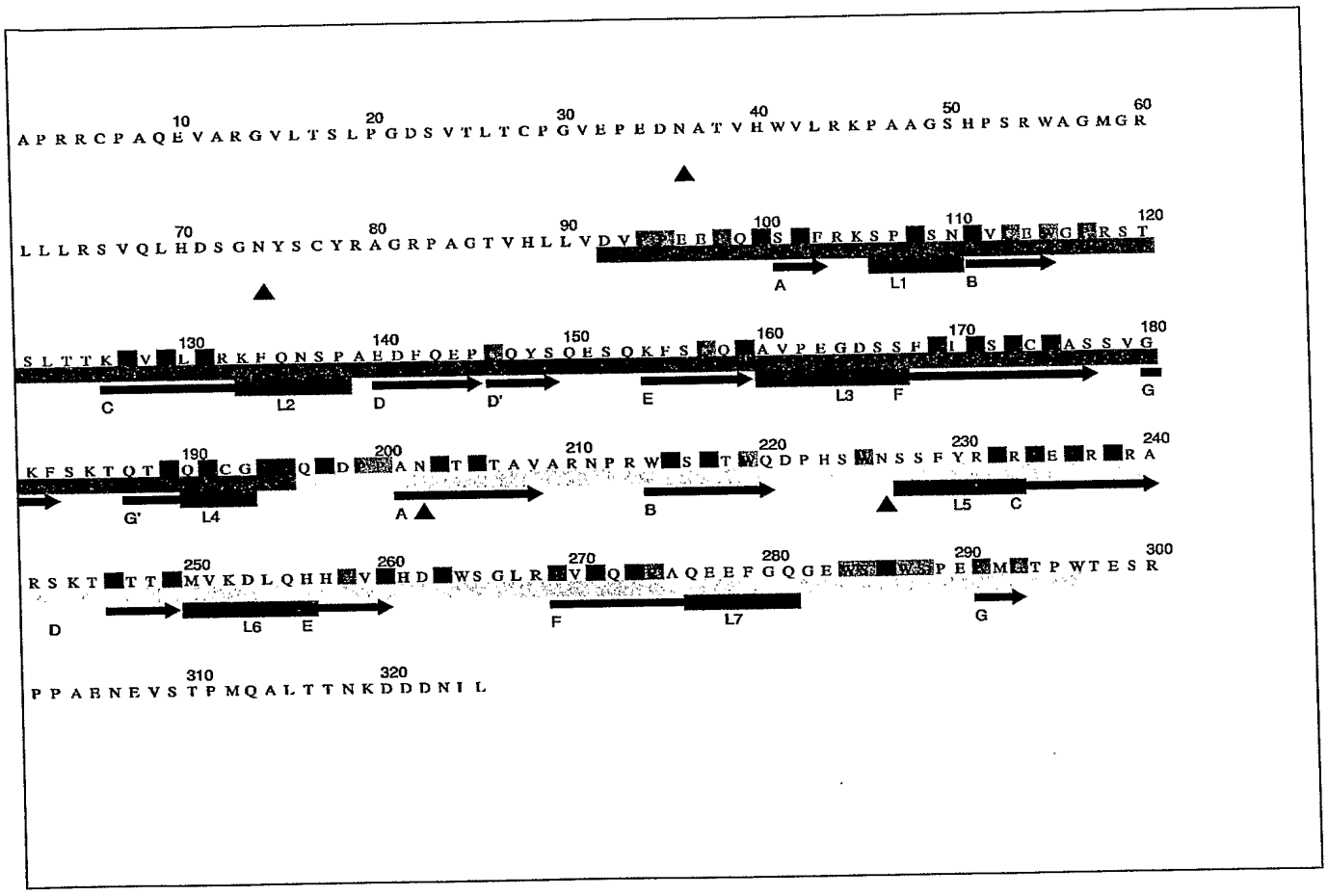
A P R R C P A Q E V A R G V L T S L P G D S V T L T C P G V E P E D N A T V H W V L R K P A A G S H P S R W A G M G R
L L L R S V Q L H D S G N Y S C Y R A G R P A G T V H L L V D V
A
L1
B
C
L2
D
D'
E
L3
F
G
K F S K T Q T Q C G Q D A N T T A V A R N P R W S T Q D P H S N S S F Y R R E R R A
G'
L4
A
B
L5
C
R S K T T T M V K D L Q H H V H D W S G L R V Q A Q E E F G Q G E P E M T P W T E S R
D
L6
E
F
L7
G
P P A R N E V S T P M Q A L T T N K D D D N I L

**Figure 3**

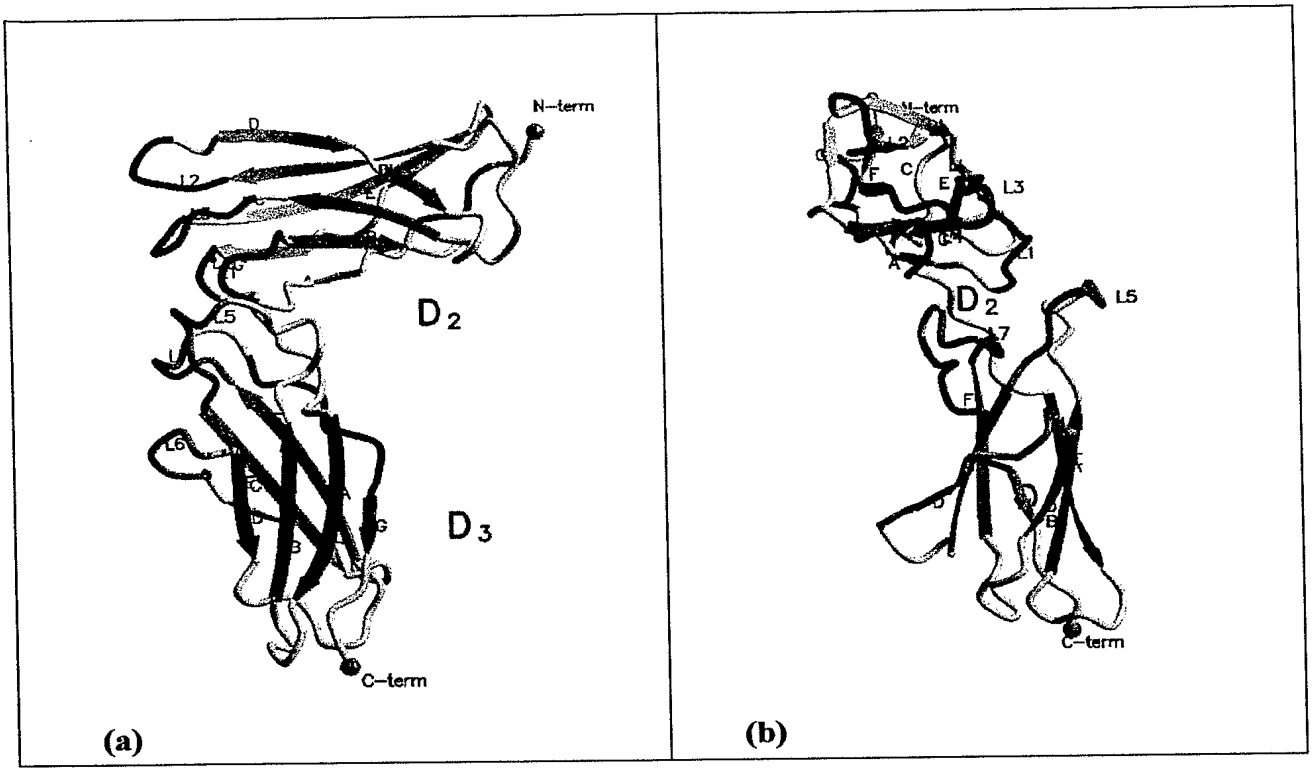


**Figure 2**

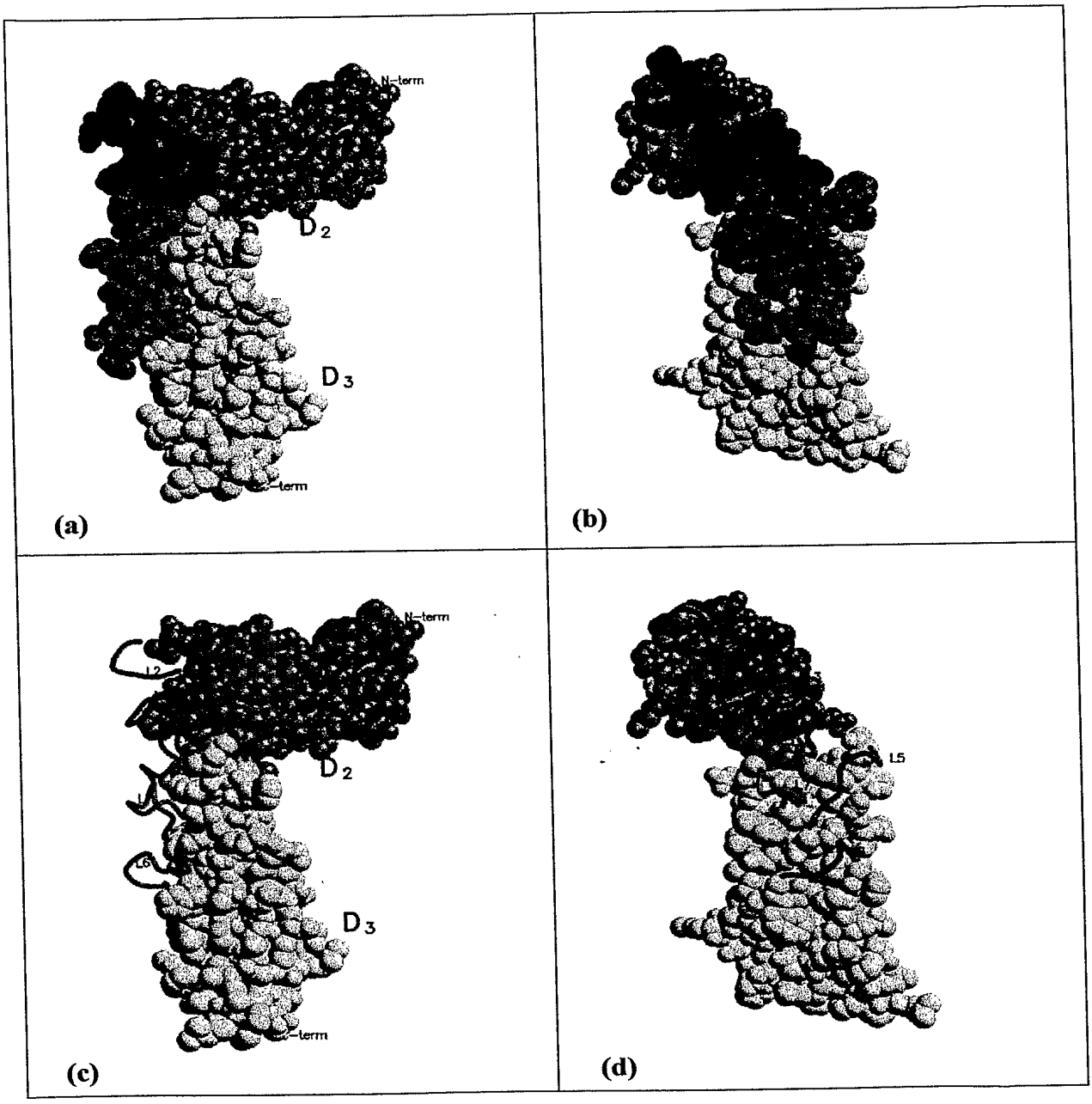


**Figure 4**

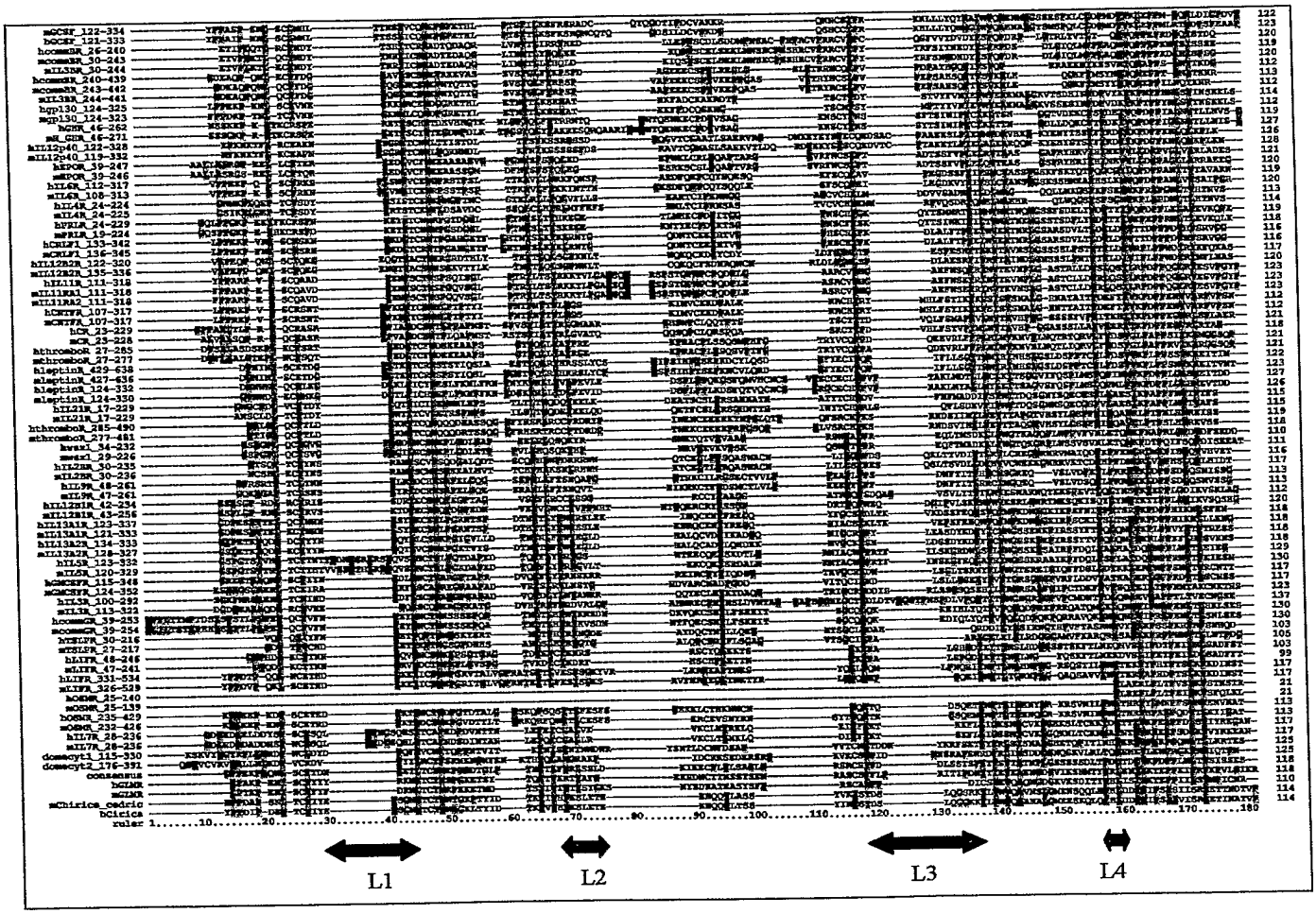


**Figure 5**

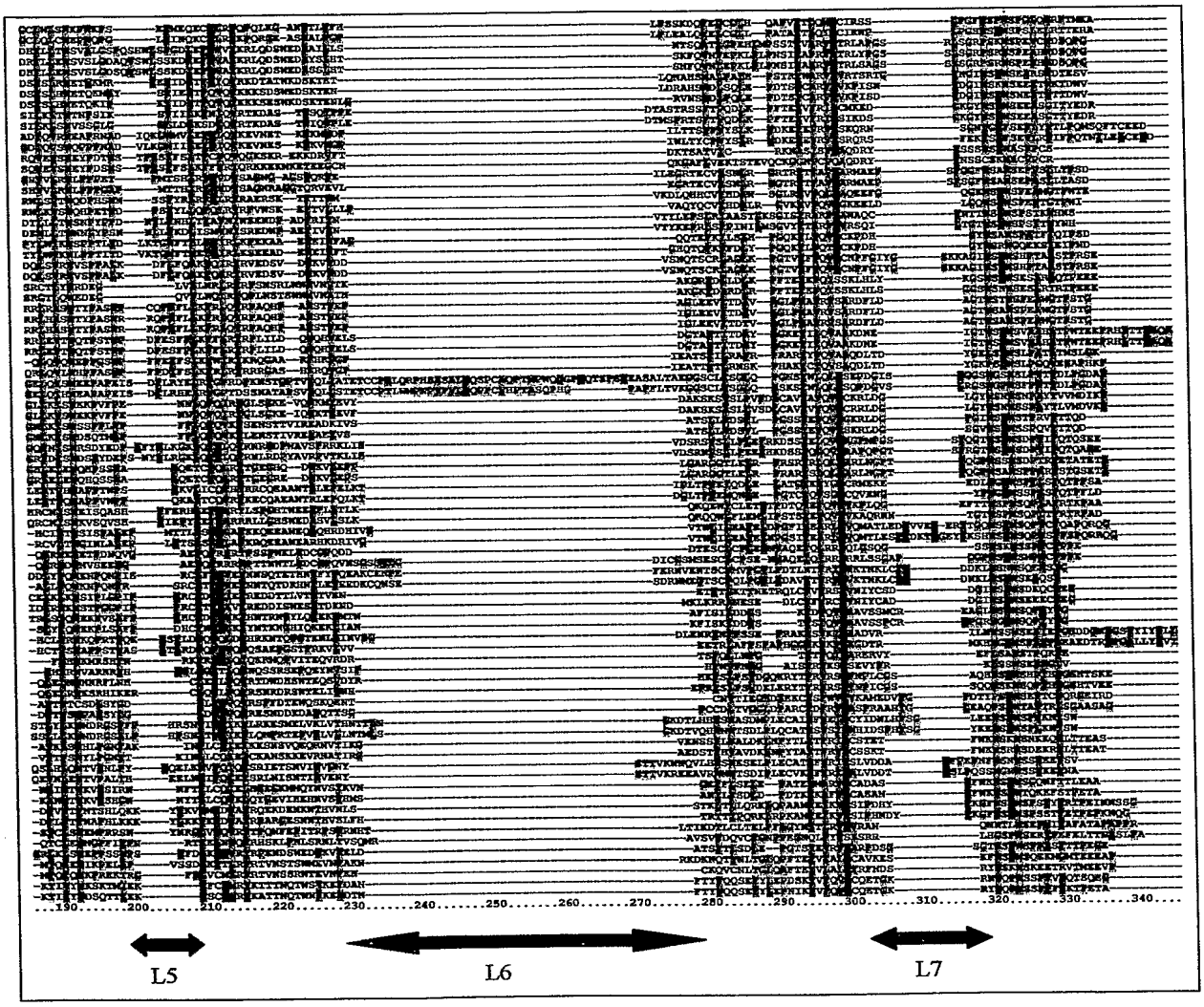


**Figure 5 (cont)**

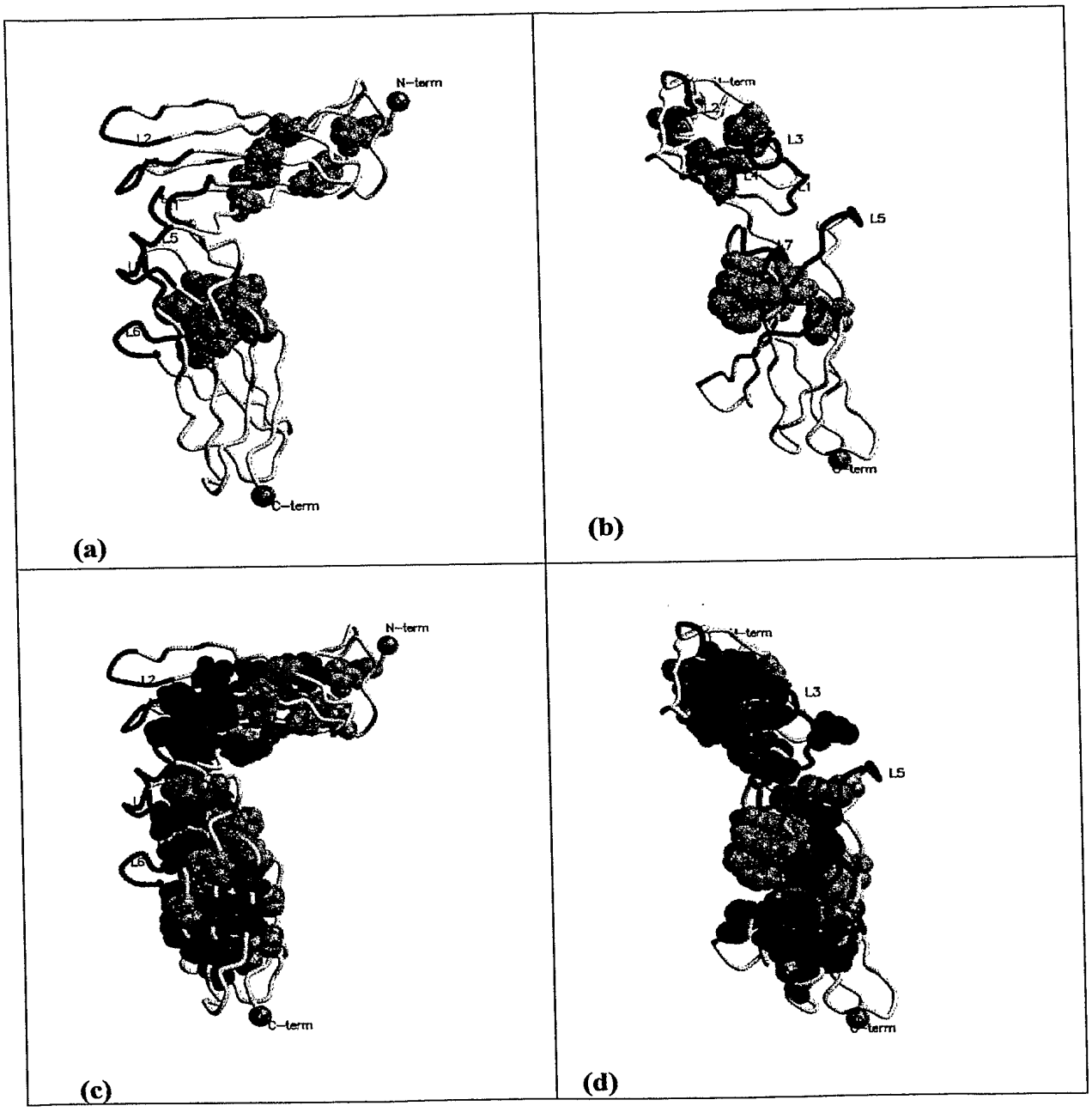


**Figure 6**

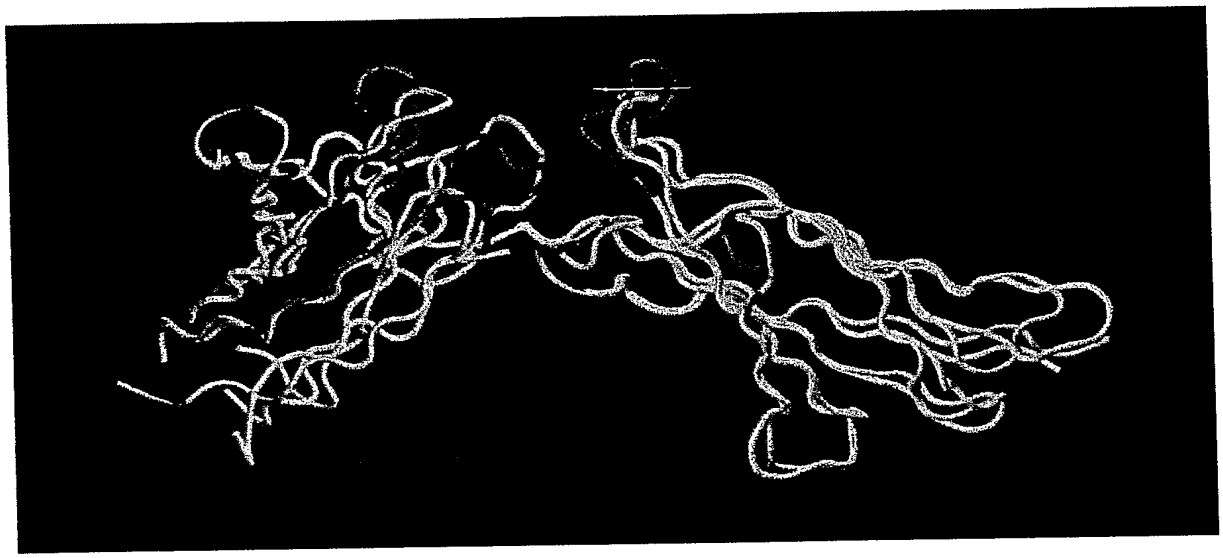

**Figure 7**